

## BEAUVERIA BASSIANA: QUERCETINASE PRODUCTION AND GENETIC DIVERSITY

Costa, Eula Maria de M. B.<sup>1</sup>; Pimenta, Fabiana Cristina<sup>2</sup>; Luz, Christian<sup>3</sup>; Oliveira, Valéria de<sup>1</sup>; Oliveira, Marília<sup>4</sup>; Bueno, Elda<sup>4</sup>; Petrofeza, Silvana<sup>4\*</sup>

<sup>1</sup>Laboratório de Bioconversão-LaBioCon, Faculdade de Farmácia, Universidade Federal de Goiás, Goiânia, GO, Brasil;

<sup>2</sup>Laboratório de Bacteriologia, Instituto de Patologia Tropical e Saúde Pública, Universidade Federal de Goiás, Goiânia, GO,

Brasil; <sup>3</sup>Laboratório de Patologia de Invertebrados, Instituto de Patologia Tropical e Saúde Pública, Universidade Federal de Goiás, Goiânia, GO, Brasil; <sup>4</sup>Laboratório de Biologia Molecular de Fungos, Instituto de Ciências Biológicas, Universidade Federal de Goiás, Goiânia, GO, Brasil.

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### ABSTRACT

*Beauveria bassiana* genetic diversity and ability to synthesize quercetin 2,3-dioxygenase (quercetinase) were analyzed. *B. bassiana* isolates, obtained from Brazilian soil samples, produced quercetinase after induction using 0.5 g/L quercetin. *B. bassiana* ATCC 7159 (29.6 nmol/mL/min) and isolate IP 11 (27.5 nmol/mL/min) showed the best performances and IP 3a (9.5 nmol/mL/min) presented the lowest level of quercetinase activity in the culture supernatant. A high level of polymorphism was detected by random amplified polymorphic DNA (RAPD) analysis. The use of internal-transcribed-spacer ribosomal region restriction fragment length polymorphism (ITS-RFLP) did not reveal characteristic markers to differentiate isolates. However, the ITS1-5.8S-ITS2 region sequence analysis provided more information on polymorphism among the isolates, allowing them to be clustered by relative similarity into three large groups. Correlation was tested according to the Person's correlation. Data of our studies showed, that lower associations among groups, level of quercetinase production, or geographical origin could be observed. This study presents the production of a novel biocatalyst by *B. bassiana* and suggests the possible industrial application of this fungal species in large-scale biotechnological manufacture of quercetinase.

**Key words:** *Beauveria bassiana*, quercetinase, quercetin-2,3-dioxygenase, genetic diversity.

### INTRODUCTION

*Beauveria bassiana* is a cosmopolitan, soil-born, and the most important entomopathogenic fungus (31, 33). Its characterization is based on isozymatic activities, entomopathogenicity, physiological characteristics, and

different DNA markers obtained by restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), internal-transcribed-spacer ribosomal region (ITS) nucleotide sequences (4, 20), and microsatellites (7). Considerable genetic variability among *B. bassiana* isolates obtained from different insects and several geographical

\*Corresponding Author. Mailing address: Laboratório de Biologia Molecular de Fungos, Instituto de Ciências Biológicas, Universidade Federal de Goiás, Goiânia, GO, Brasil.; E-mail: [petrofez@uol.com.br](mailto:petrofez@uol.com.br)

origins has been described (6, 10).

*B. bassiana* secretes toxic proteins, such as beauvericin and oosporein (14). A toxic metabolite, named bassiacridin, and 19 enzymes produced by *B. bassiana* EABb 90/2-Dm, detected by API-ZYM<sup>TM</sup> (bioMérieux, Marcy l'Étoile, France) system, have also been described (23).

Quercetin is a flavonoid that occurs widely in plants and is generally known to have a broad set of pharmacological effects on human health, including antimicrobial properties (13, 21). This compound is degraded by different fungi species in their extracellular enzyme system (27). In these organisms, quercetin-2,3-dioxygenase or quercetinase (EC.1.13.11.24) catalyzes the oxidative transformation of quercetin into 2-protocatechuoylphloroglucinol carboxylic acid by the insertion of molecular oxygen with the concomitant production of carbon monoxide (12, 16, 29).

A combination of screening for enzymes and biotechnology opens new and important possibilities for the industrial and commercial use of microbial enzymes (18). Quercetinase production has been reported for several fungi and any bacteria species, among which the most important are *Aspergillus japonicus*, *A. flavus*, *A. awamori*, *A. niger*, *A. foetidus*, *A. oryzae*, *A. soyae*, *Penicillium minioluteum*, *P. roquefortii*, *P. olsonii*, *Bacillus subtilis*, *Diapotha eres*, *Neurospora crassa*, and *Diplodia gossypin* (27, 29). However, as far as we are concerned, no information on the production of quercetinase by *B. bassiana* is currently available.

This study aimed to verify quercetinase production by the isolates of *B. bassiana*, investigate the genetic variability of these isolates, and verify a possible correlation between genetic diversity and quercetinase production so that the results can improve our knowledge on *B. bassiana* enzyme production and help establish a novel biotechnological use for this fungus.

## MATERIALS AND METHODS

### Microorganisms

A total of 10 isolates of *B. bassiana* obtained from

Brazilian Cerrado soils plus the reference isolate ATCC 7159 strain were used in this study. The samples were collected in 2001 in natural reserves as follows: isolates IP 3a, IP 6, IP 8, IP 11, and IP 94 – Parque Nacional das Emas (17°50'–18°15'S, 52°30'–53°10'W) near the municipality of Chapadão do Céu; isolates IP 98, IP 129, and IP 132 – Chapada dos Veadeiros (13°51'–14°10'S, 47°25'–47°42'W) near the municipality of Alto Paraíso; isolates IP 147 and IP 153 – Estação Florestal do IBAMA (11°40'–55°7'S, 49°04'–03°09'W) near the municipality of Silvânia, all of them located in the state of Goiás, in the Midwestern Region of Brazil. The minimum distance between sampling areas was 350 km. The isolates were maintained in potato-dextrose agar (PDA) and stored in refrigerator at 4°C until further use.

### Induction of quercetinase production

A conidial suspension of each isolate (final concentration –  $10^6$  conidia/L), prepared in 25% (v/v) sterile glycerol to 7-day-old grown *B. bassiana* PDA plates, was inoculated into 100 mL of basic liquid medium (16) and incubated at 29°C for 3 days on an orbital shaker at 200 rpm. Quercetinase production was induced at the end of the fungus exponential growth phase. To test quercetin as an inducer, this compound was dissolved in 5 mL of dimethylsulfoxide (DMSO) to reach a final concentration of 0.5 g/L and aliquots of the culture were taken for analysis every 24 h for 4 days. All the experiments were carried out in triplicate. In the control experiment, the inducer was replaced with 5 mL of DMSO.

Optimal fungal growth had been previously established by our research group for *B. bassiana* (strain ATCC 7159) under non-induced (basal medium, no inducer) and induced (quercetin 0.5 g/L) conditions.

### Enzyme assay

Quercetinase activity was determined by monitoring the decrease in the absorbance peak at 367 nm due to quercetin decomposition (3, 15) using a standard procedure (29). Briefly, the reaction was conducted at 25 °C in 3 mL of MES buffer

(0.1 M, pH 6) containing 30  $\mu$ L of a 10mM stock solution of quercetin in dimethyl sulfoxide and 30  $\mu$ L of centrifuged culture supernatant. Enzyme activities were expressed as specific activity (units  $\text{mg}^{-1}$  protein). Protein assays were performed using the Protein Assay kit (Quant-iT -Invitrogen). One unit of quercetinase was defined as the amount of enzyme oxidizing 1  $\mu$ mol of quercetin per min.

### Genotypic characterization

The extraction of genomic DNA from all the 11 isolates of *B. bassiana* was performed according to the CTAB method (5).

### RAPD-PCR analysis

RAPD analysis of the isolates was carried out using 16 primers OPC, OPE, and OPW (Operon Technologies, Inc., Alameda, CA, USA). We prepared a final reaction volume of 30  $\mu$ L containing 10 ng of template genomic DNA, 2.5 mM of each of four dNTPs, 2.5 U *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA), 1.5 mM  $\text{MgCl}_2$ , 10X buffer. PCR amplification was performed using a DNA thermocycler PTC 100 MJ with the following temperature profiles: 1 cycle at 95°C for 5 min; 35 cycles at 94°C for 1 min; 45°C for 1 min; 72°C for 2 min; and 1 cycle at 72°C for 5 min. To ensure the reproducibility, PCR reactions for each isolate were performed in duplicate. RAPD-PCR products were separated by electrophoresis in 1.5% agarose gel using TBE buffer (2 mM EDTA; 0.1 M Tris-HCl; 0.1 M boric acid, pH 8.0) at 4 V/cm, visualized under UV light after ethidium bromide staining (0.5  $\mu\text{g/mL}$ ), and photographed using the Eagle Eye II photodocumentation system (Stratagene, New Jersey, USA). The  $\lambda$  phage DNA digested with *Eco*RI and *Hind*III was used as a molecular weight marker.

### Data analysis

The RAPD bands were scored for their presence (1) or absence (0) at positions and the scores were assembled in a rectangular data matrix. Bands of the same size obtained by the same primer were scored as identical and only the ones repeated in at least two different PCR amplifications with the

same primer were evaluated. Dendrogram constructions were performed by cluster analysis using Jaccard's coefficient and the unweighted pair-group method with arithmetic average (UPGMA). Person's correlation coefficient (r) was estimated among the matrix of genetic distance (1 – index of similarity of Jaccard) and of geographical distances or level of quercetinase production. These data were analyzed using the NTSYSpc (Numerical Taxonomy and Multivariate Analysis System) package, Version 2.2 (24).

### ITS-RFLP and ITS sequencing

A fragment of the ITS spacer region (ITS1 region, 5.8S gene and ITS2 region) was amplified by PCR utilizing the universal oligonucleotides ITS5 (5'GGAAGTAAAAGTCGTA ACAA-3') and ITS4 (5'TCCTCGCTTATTGATATGA-3') (34). The reaction consisted of 0.25  $\mu\text{M}$  of each oligonucleotide, 0.2  $\mu\text{M}$  of each dNTP, 1.5  $\mu\text{M}$   $\text{MgCl}_2$ , 50 mM KCl, 10 mM Tris-HCl pH 8.4, 2.5 U *Taq* DNA polymerase (Invitrogen), 10 ng of genomic DNA, and Milli-Q  $\text{H}_2\text{O}$  to a final volume of 30  $\mu\text{L}$ . Amplification was carried out in a PTC 100 thermocycler, programmed for a cycle of 94°C for 3 min, followed by 40 cycles at 92°C for 1.5 min, 42°C for 1.5 min, 72°C for 2.5 min, and a final extension at 72°C for 5 min. The size and quality of the PCR product were determined by electrophoresis in 0.8% agarose gel stained with ethidium bromide (0.5  $\mu\text{g/mL}$ ) and visualized under UV light (25). The DNA size marker employed was  $\lambda$  phage DNA digested with *Eco*RI and *Hind*III (Gibco/BRL).

The PCR products of the ITS regions of the 11 isolates were digested according to the manufacturer's recommendations, using 5  $\mu\text{L}$  of each PCR product (300 ng) and 5 to 10 units of the following restriction enzymes: *Msp* I, *Hae* III, *Taq* I, and *Ava* I. The restriction fragments were separated in 1.5% agarose gel, stained with ethidium bromide (0.5  $\mu\text{g/mL}$ ), and visualized under UV light. The size of the fragments was estimated based on 100 bp size markers (Promega).

After the analyses, the PCR products were sequenced directly according to the manufacturer's instructions using a MegaBace 1000 automatic sequencer (Amersham Biosciences, Piscataway, NJ, USA). Each reaction mixture comprised 1  $\mu$ L of ITS-PCR product (100 ng/ $\mu$ L), 4  $\mu$ L of DYEnamic Kit ET DYE (Terminator Cycle Sequencing for MegaBace; Pharmacia Biotech, Denver, CO), 0.5  $\mu$ L of oligonucleotide (0.5  $\mu$ M), and Mili-Q water to a final volume of 10  $\mu$ L. The amplification reactions were performed in a 96-well GeneAmp PCR System 9700 thermocycler (Applied Biosystem, Foster City, CA, USA) under the following conditions: initial denaturation at 96°C for 2 min, 35 cycles at 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. The quality of the chromatograms obtained was examined using the Phred program (8). The sequences generated were compared with existing data available in GenBank using the similarity search program Basic Local Alignment Search Tool (BLAST) (1,2), thereby exploring available sequences of DNA databanks. The sequences belonging to isolates classified as *Beauveria* showing Phred quality scores > 20 were aligned using the ClustalW program, and the genetic distance was calculated using the Kimura coefficient (17). The phylogenetic relationship among the nucleotide sequences of the isolates was determined using the phylogeny inference package (PHYLIP) program, version 3.5 (28). The confidence levels for the generated groupings were determined by bootstrap analysis (1000 repetitions) (9).

## RESULTS

### Enzyme assay

In this study, we focused only on quercetinase activity in the supernatant fraction, since we assumed that this activity would be more relevant to the role of quercetinase during the quercetin biotransformation process in order to obtain derivatives for pharmaceutical use.

All isolates tested, including the reference strain, *B. bassiana* ATCC 7159, were able to produce quercetinase when grown on culture medium supplemented with quercetin.

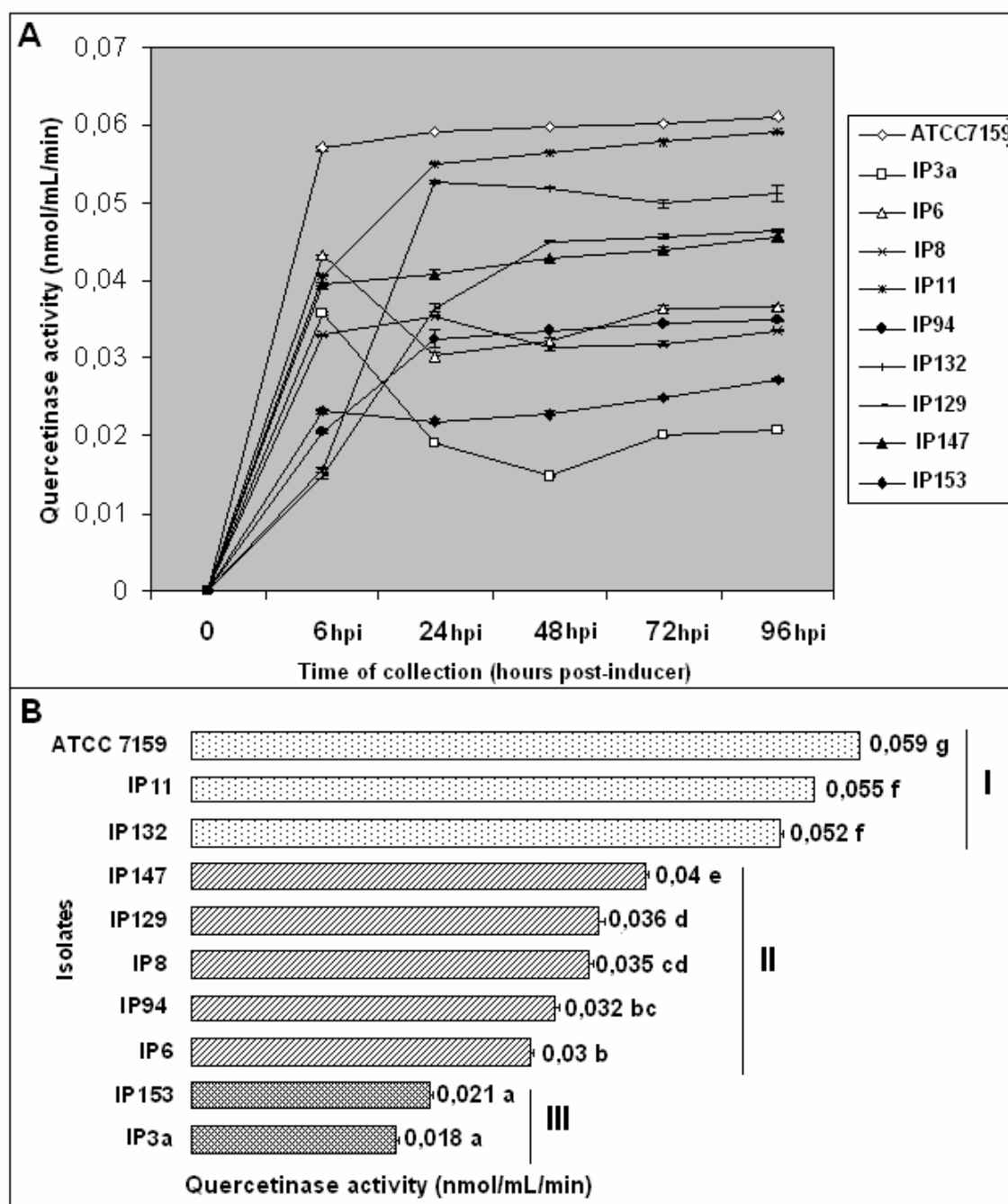
Quercetinase production was monitored for a period of 96 h after induction. The accumulated enzyme activities showed markedly different patterns among the *B. bassiana* isolates tested. The analysis of variance of the scores given to the quercetinase production is shown in Table 1. A significant difference was detected among isolates ( $P < 0.01$ ). Quercetinase activity varied by a factor of approximately 3 between the isolates under cultivation conditions. *B. bassiana* ATCC 7159 and IP 11 showed the highest levels of quercetinase activity and the isolate IP 3a showed the lowest one.

The highest level of quercetinase production was observed within the period of 24 h after induction with quercetin (Figure 1A). Considering this time, the isolates were separated into groups based on their quercetinase activity level: Group I, comprising high-productive isolates, ATCC 7159 (29.6 nmol/mL/min), IP 11 (27.5 nmol/mL/min), and IP 132 (26.4 nmol/mL/min); Group II, comprising isolates that presented an intermediary level of quercetinase production, IP 147 (20.4 nmol/mL/min), IP 129 (18.3 nmol/mL/min), IP 8 (18.2 nmol/mL/min), IP 94 (16.3 nmol/mL/min), and IP 6 (15.1 nmol/mL/min); and Group III, comprising low-productive isolates, IP 153 (10.9 nmol/mL/min) and IP 3a (9.5 nmol/mL/min) (Figure 1B). The Tukey test clearly showed that the patterns of enzyme production vary among isolates, but a specific pattern may also be shared by several isolates (Figure 1B). This evaluation time was chosen considering previous studies carried out by our group, which demonstrated drastic decrease in quercetin in the supernatant 24 h after the quercetin biotransformation process started.

**Table 1.** Analysis of variance for quercetinase production in the *B. bassiana* isolates.

Summary growth AV	DF	Sum Sq	Mean Sq	F value
Strains	10	0.00507	0.00056	432.2403 *
Residuals	20	0.00003	0.00000	
Total	30	0.00510		

\*Significant at 1% probability by the F test.



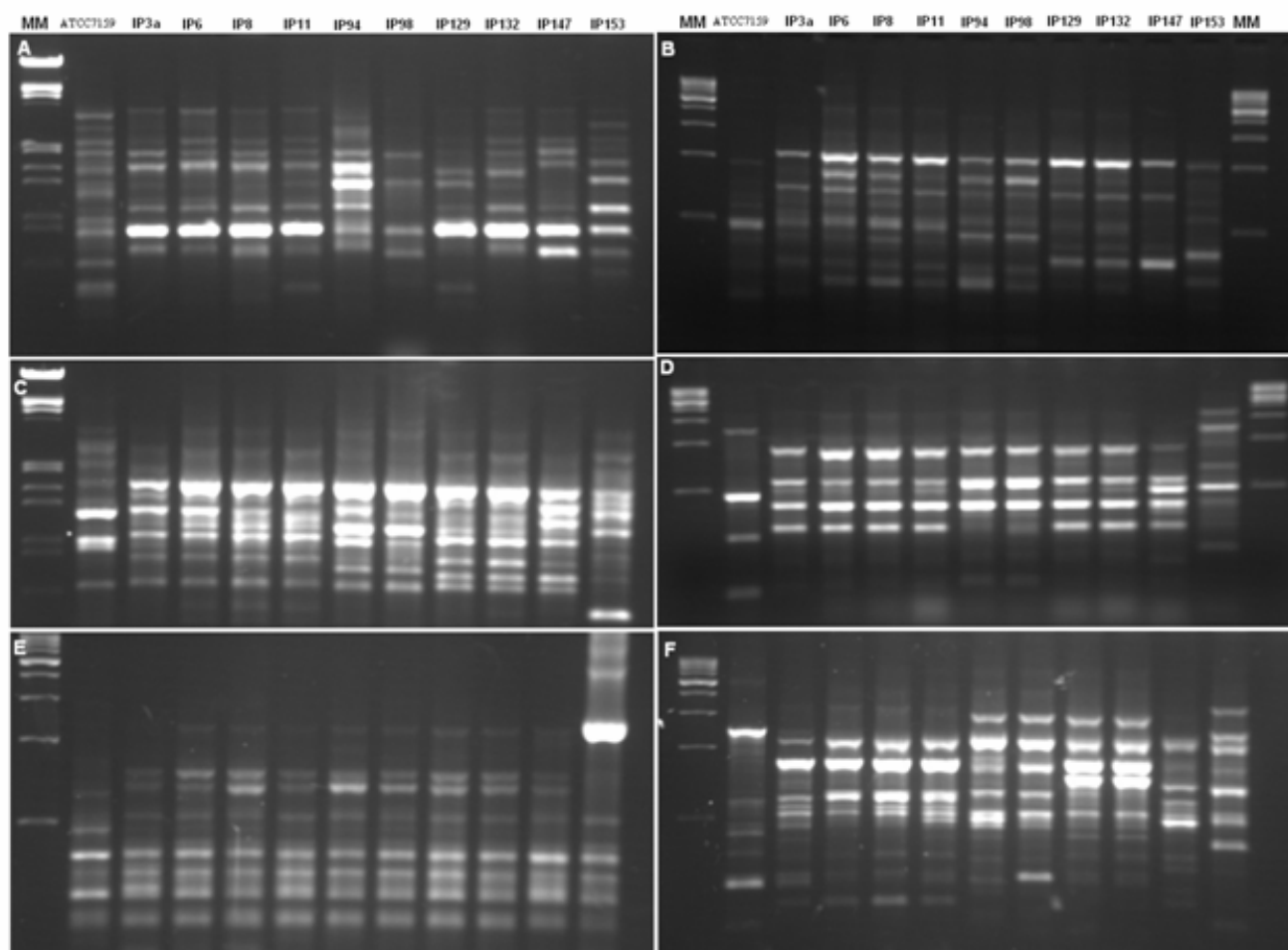
**Figure 1.** Analysis of extracellular production of quercetinase activity available in the culture supernatant of *Beauveria bassiana*. (A) Time course curves of quercetinase activity produced by different isolates of *B. bassiana* during growth after induction using 0.5 g/L quercetin; (B) Quercetinase activity assay 24 h post-induction with quercetin. Group I – isolates presenting the highest level of quercetinase production; Group II – isolates presenting an intermediary level of quercetinase production; and Group III – isolates presenting the lowest level of quercetinase production. Each data point is the average of three independent experiments and bars represent the standard deviation from the mean. Means followed by the same letter are not significantly different according to the Tukey test at  $P < 0.05$ .

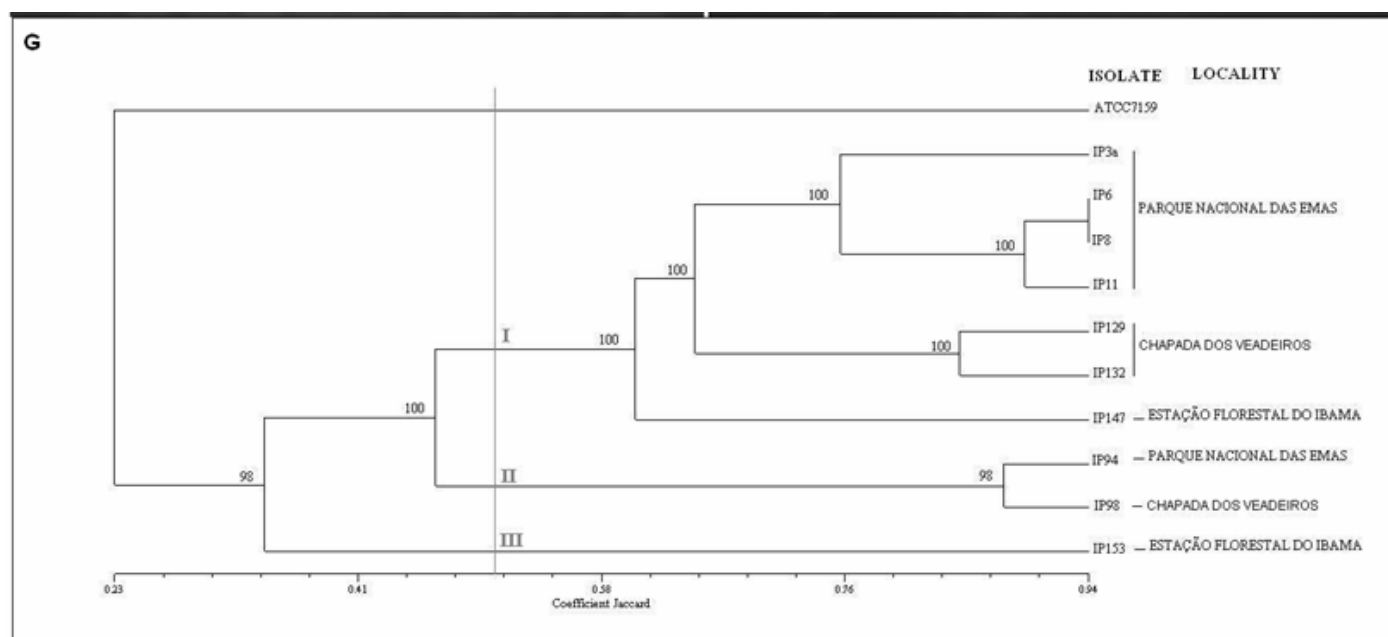
### Genotypic characterization

**RAPD-PCR analysis:** We selected 11 out of the 16 arbitrary primers tested since they generated reproducible banding patterns for RAPD-PCR. A total of 164 distinct bands, consistent with repeated amplifications, were amplified using the DNAs from the 11 *B. bassiana* isolates as templates. Depending on the isolate–primer combination, the bands were scored ranging in size from 0.4 kb to 3.2 kb among the isolates of *B. bassiana* collected from different geographical areas (Figure 2).

Distinct fingerprint types were observed for isolates

varying in their geographical origin. Based on the similarity values, a matrix of similarity was obtained. UPGMA clustering was conducted to graphically display the possible groups. The dendrogram clearly shows that the isolates examined can be divided into three distinct groups at 50% similarity, designated I, II, and III, and it also reveals a high genetic diversity among these groups (Figure 2). Reliability estimates calculated by bootstrapping with high values for each cluster and a cophenetic correlation of 0.956 confirmed that the groups were distinct.





**Figure 2.** Agarose electrophoresis showing the random amplified polymorphic DNA (RAPD) and dendrogram constructed with UPGMA clustering method among isolates of *Beauveria sp* and *B. bassiana* ATCC 7159. Banding patterns amplified with six of the arbitrary primers used in this study: (A) Primer OPE 02; (B) Primer OPE 07; (C) Primer OPE 11; (D) Primer OPE 12; (E) Primer OPE 14; (F) Primer OPE 18. Lane 1 represents *Beauveria bassiana* ATCC 7159 and lanes 2 to 11 represent the isolates of *Beauveria bassiana*, IP3a, IP6, IP8, IP11, IP94, IP98, IP129, IP132, IP147, IP153, respectively; MM – molecular marker: *Eco* RI and *Hind* III digested phage  $\lambda$  DNA; Similarities in dendrogram were computed from 164 random amplified polymorphic DNA loci (G). The scale in the dendrogram is the genetic similarity coefficient calculated according to Jaccard. Numbers where the notes represent 1,000 replications were bootstrap-generated using the program WinBoot (32).

### ITS-RFLP analysis and ITS sequencing

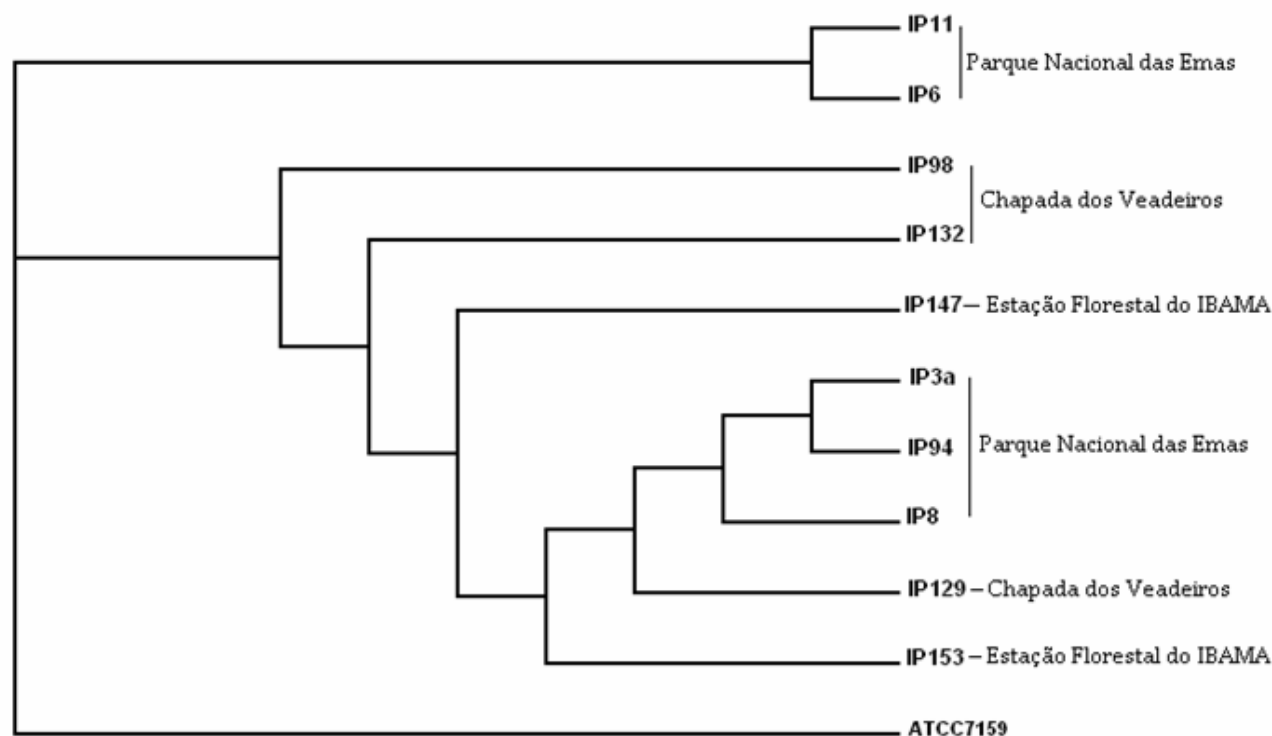
The ITS4 and ITS5 primers amplification for the 11 *B. bassiana* isolates tested resulted in a single product of approximately 600 bp, which was digested with four restriction enzymes. Two to five restriction fragments per PCR product were obtained among the 11 isolates depending on the enzyme used (data not shown). *Ava* I had no site in the amplified fragment. *Hae* III presented similar restriction fragments pattern for 9 isolates, and only isolates IP 147 and IP 153 showed different patterns. *Msp* I also presented similar restriction fragments pattern for 9 out of the 11 isolates, and *Taq* I digestion resulted in the same restriction pattern for all isolates.

The alignment of the partial sequences corresponding to the ITS1 region, 5.8S gene, and ITS2 region revealed similarity among the 11 isolates. The highest similarity was observed in the region corresponding to the 5.8S gene, and the highest diversity, in the corresponding ITS1 and ITS2 regions.

The genetic distance analysis permitted the distribution of the isolates into three groups (Figure 3), and those presenting the same restriction profile, determined by PCR-RFLP, showed variability in the sequence. These results suggest that ITS-RFLP does not provide characteristic markers for differentiating isolates of *B. bassiana*.

With the purpose of analyzing patterns of space variation, Person's correlation coefficient ( $r$ ) was estimated among the

matrix of genetic distance and of geographical distances or level of quercetinase production, and a lower correlation ( $r = 0.003$ , and  $r = -0.024$ ,  $p < 0.001$ ) was found, respectively.



**Figure 3.** Neighbor-joining tree generated by Clustal X for the 11 partial sequences ITS1-5.8S-ITS2 regions of *Beauveria bassiana*. The sequences have been deposited in the GenBank and their accession numbers are in parentheses: IP 3a (FJ827072), IP 6 (FJ827070), IP 8 (FJ827075), IP 11 (FJ827071), IP 94 (FJ827074), IP 98 (FJ827076), IP 129 (FJ827073), IP 132 (FJ827077), IP 147 (FJ827079), IP 153 (FJ827078).

## DISCUSSION

To the best of our knowledge, quercetinase production by *B. bassiana* has been herein reported for the first time. All isolates investigated presented quercetinase activity using quercetin as potential quercetinase inducer. A study on inducers of quercetinase activity in *Penicillium olsonii* concluded that rutin, quercetin, and phloroglucinol carboxylic acid act as quercetinase inducers (30).

In our study, *B. bassiana* isolates presented different quercetinase activities. The kinetics of quercetinase production

by *P. olsonii* was fully expressed 4 days post-induction (29), while for *B. bassiana* we observed rapid quercetinase secretion between 6 h to 24 h after induction, possibly because of quercetin antimicrobial properties (13), since rapid quercetinase production by microorganisms could be a self-protection mechanism in response to the antimicrobial activity this flavonoid exhibits (19).

*B. bassiana* strain ATCC 7159 presented only 23% genetic similarity with the isolates from Brazilian soils. At the enzyme production levels, this fungus demonstrated a pattern distinct from the other isolates and showed the highest level of



quercetinase synthesis. The reasons for these facts remain unknown and should be investigated.

All the 11 isolates of *B. bassiana* presented high level of polymorphism based on RAPD-PCR analysis, which reflects a considerable genetic variability among isolates from the same geographical area or not. Our results are in agreement with data reported for the *Beauveria* genus fungi isolated in South America, which presented considerable genetic diversity (11), and are also in accordance with the high variability amongst *B. bassiana* populations from natural reserves already observed (33). It seems that isolates from natural reserves present considerable genetic diversity due to multiplicity of habitats and hosts.

In contrast with RAPD, the results obtained for ITS-RFLP did not provide characteristic markers to differentiate the isolates, probably because there is not enough variation in the region analyzed by the restriction enzymes tested. This lack of correlation could be explained by the fact that the primers and the restriction enzymes used in different molecular typing methods detect polymorphisms in different regions of the genome.

The ITS primers utilized in this study amplified a fragment with approximately 600 bp for all isolates, data also reported for *B. bassiana* collected in different Brazilian regions (26). Analysis at nucleotide level provided more informative and discriminatory results and the ITS sequences from all strains suggested that the isolates examined could have a common genetic basis with low variation at conserved regions. ITS sequences confirmed the identity of the isolates analyzed and discriminated them in three groups.

The genetic analysis, RAPD, and ITS did not provide a correlation between the geographical origin of the isolates and their genetic diversity. These observations are in consonance with data described in the literature (10).

Genetic variability among *Beauveria* species has been associated with toxin production and enzymatic activity (22). In our experiment, the isolates were clustered according to their genetic diversity and lower association, according to the

Person's correlation, could be established between the groups and their quercetinase production potential or geographical origin.

Future industrial-scale application of *B. bassiana* in biotechnological manufacturing of quercetinase is promising. Our results present a novel biocatalyst synthesized by *B. bassiana* and show that this can be a remarkable way to produce quercetinase using a biotechnological process.



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